Cloning and expression of the *Escherichia coli* replication origin in a single-stranded DNA phage

(M13/rep/oriC/bidirectional replication)

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**ABSTRACT** The *Escherichia coli* DNA replication origin (oriC) and the adjacent asparagine synthetase gene (asnA) have been inserted into the duplex replicative form DNA of the single-stranded phage vector M13Gor1. By *in vitro* recombination, the entire *oriC* asnA-containing plasmid pJS5 was inserted into M13Gor1 in both possible orientations. Both phage types transduce the *asnA* gene and confer upon the M13 vector the ability to replicate as a plasmid in the *E. coli* mutant rep2. In rep+ hosts, these phages undergo single-stranded DNA synthesis and viral morphogenesis.

The mechanism by which the strands of duplex DNA are separated to serve as template during *Escherichia coli* chromosomal replication is unknown. Recent studies have indicated that a host enzyme, the *rep* protein, functions in this strand separation process during replication of the single-stranded DNA phage φX174 (1, 2). A similar function has been proposed for *rep* protein in the replication of the *E. coli* chromosome. However, a mutation in the *E. coli* *rep* gene (3), that prevents replication of the duplex replicative form (RF) of the single-stranded DNA phages φX174, G4, and M13 has little effect on the replication of the bacterial chromosome (4). Does this striking difference in the requirement for the *rep* function relate to the considerably greater structural and topological complexity of the bacterial chromosome or possibly to a different location of the viral DNA within the cell? We have addressed these questions by inserting the *E. coli* replication origin (oriC) into the chromosome of an M13 phage vector and investigated its replication in an *E. coli* rep mutant.

The mutant rep2 was isolated by Denhardt et al. (3) on the basis of its resistance to φX174-type bacteriophages (5). It was also found to be unable to propagate the unrelated filamentous single-stranded DNA phages of the M13 type. Analysis of viral DNA synthesized in *E. coli* rep2 revealed that the infectious process of the single-stranded DNA phages φX174, G4, and M13 is blocked at a specific step in the DNA replication cycle, the replication of the parental RF (5–7). In each case, the infecting single-stranded circular viral DNA was converted to RF and specifically nicked in the viral strand preparatory to initiating replication of the viral strand, presumably by a rolling circle mechanism (8). The nicking event has been shown to be mediated by the only phage-specific protein directly required for replication of the parental RF. In the case of φX174 and presumably G4, this protein is encoded by gene A (9, 10). In the case of M13, this protein is encoded by gene 2 (11). Replication beyond this point is inhibited, and essentially all of the viral DNA accumulates as the parental RF, containing the infecting viral strand and its newly formed complementary strand. The block in phage replication due to the absence of *rep* function thus appears to be at a step subsequent to nicking of the viral strand.

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Studies on φX174 RF replication in *vitro* have shown that, in the presence of the *φX174* gene A protein, *rep* protein, DNA polymerase III holoenzyme, and DNA binding protein, viral DNA circles are synthesized on φX174 RF template in excess of the input template RF (1). It has been shown that purified *rep* protein is associated with a single-stranded DNA-dependent ATPase activity. In addition, *rep* protein is capable of strand separation of the proper duplex DNA substrate in the presence (1) or absence (2) of gene A protein, DNA binding protein, Mg2+, and ATP. It is thus thought that *rep* protein functions in duplex replication during the process of elongation by providing the activity required to unwind the helix of duplex DNA.

We have sought to study more closely the involvement of *rep* protein in DNA replication under conditions uncomplicated by the structural complexity of the bacterial chromosome. The results presented here indicate that *oriC*-dependent replication still occurs in a rep+ host even when *oriC* is contained within a small viral DNA molecule that otherwise requires the *rep* function for even a single round of duplex DNA replication.

**MATERIALS AND METHODS**

**Bacterial and Phage Strains.** The bacterial strains used are derived from *E. coli* K-12. Their origin and genotypes are given in Table 1. The construction of the M13Gor1 phage vector and M13::Tn3-15 has been described (12, 13).

**Microbiological Procedures.** P1 *vir* stocks were prepared and P1 transductions were performed as described (14). Stocks of the chimeric phages were prepared from single plaques by using *E. coli* K-37 as the host as described (14). Because the viral DNAs contain *E. coli* K restriction/modification sites, K-37, which is *rX*+, *mK*+, was used as host for preparation of phage stocks. YT broth, L broth, minimal A glucose medium, and plates were prepared as described (14).

**Enzymes.** Hae III restriction endonuclease was prepared by a slight modification of the technique described by Roberts et al. (15). *Hpa* II was purchased from Worthington. *Xho* I and T4 DNA ligase were purchased from Bethesda Research Laboratories, Rockville, MD.

**Construction and Isolation of M13oriC26 and M13oriC81.** The *oriC* plasmid pJS5 is a derivative of pSY221 (16) containing only DNA sequences from the *oriC* asnA region of the *E. coli* chromosome and a single *Xho* I cleavage site (J. F. Scott and S. Yasuda, personal communication). This single *Xho* I site delimited one boundary of the *oriC* region (16, 17). pJS5 circular DNA [3550 base pairs (bp)] was cleaved with *Xho* I to produce unit-length linear molecules. The M13 vector M13Gor1 contains a single *Xho* I cleavage site within the cloned G4 sequence in the region of the G4 complementary strand origin (18–20). M13Gor1 RF was also cleaved with *Xho* I to produce unit-long linear molecules.
length linear molecules and was combined with Xho I-digested pJS5 DNA. The mixture was treated with T4 DNA ligase under conditions described by de Vries et al. (21). Calcium chloride-treated E. coli LA-6 was then transfected with the ligated DNA (22) and plated for M13 plaque formation. The resulting plaques were screened by plaque hybridization (23). Plaques containing DNA complementary to the pJS5 DNA probe labeled by nick translation were isolated. RF DNA from these isolates was prepared (24) and analyzed by restriction and agarose gel electrophoresis (25).

Restriction Endonuclease Digestion of DNA and Gel Electrophoresis. Digestions were performed at 37°C for 2 hr with sufficient enzyme to give complete digestion. Hae III buffer contained 6 mM Tris-HCl (pH 7.5), 6 mM NaCl, 6 mM MgCl₂, 6 mM mercaptoethanol, and 100 μg of gelatin per ml. Hpa II buffer contained 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 6 mM KCl, 1 mM dithiothreitol, and 100 μg of gelatin per ml. Digestion mixtures were placed on ice after incubation and made 0.1% in bromphenol blue and 5% (wt/vol) in glycerol prior to electrophoresis. They were then layered onto a 4% vertical polyacrylamide slab gel, electrophoresed, stained with ethidium bromide, and photographed (25). Unrestricted DNA isolated from a crude lysate (24) was analyzed by gel electrophoresis on a 0.7% horizontal agarose gel, stained with ethidium bromide, and photographed (25).

RESULTS

Characterization of M13oriC26 and M13oriC81 RFI by Restriction Enzyme Cleavage. To establish clearly the identity and orientation of the cloned DNA, the recombinant DNAs were cleaved with Hae III or Hpa II. When M13oriC26 RFI or M13oriC81 RFI was cleaved with Hae III, the resulting restriction patterns (Fig. 1, lanes 3 and 4) showed bands that comigrated with those of both Hae III-cleaved M13Gori1 supercoiled RF (RFI) (lane 1) and Hae III-cleaved pJS5 (lane 2). The doublet band of lane 1 migrating at the position of a 440-bp fragment appeared as a singlet band in the corresponding positions of lanes 3 and 4. This result indicates that the site of insertion of pJS5 into M13Gori1 is within one of the restriction fragments of the doublet, as expected from the known location of the single Xho I site. The Hae III fragment of greatest molecular weight of pJS5 shown in lane 2 of Fig. 1 was absent in the banding patterns of lanes 3 and 4, indicating that this DNA fragment contains the Xho I site where insertion of M13Gori1 into pJS5 occurred. Two new bands appeared in the Hae III digests of M13oriC26 RFI and M13oriC81 RFI. The DNA of these bands contained the junctions where pJS5 sequences joined M13Gori1 sequences. The fact that these junction fragments are not identical in M13oriC26 and M13oriC81 indicates that the inserted pJS5 plasmid is inserted into the M13Gori1 genome in opposite orientations.

The cleavage patterns obtained when M13Gori1 RFI and pJS5 were cleaved with Hpa II are shown in lanes 5 and 6 of

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Table 1. E. coli K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or construction</th>
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</thead>
<tbody>
<tr>
<td>ER</td>
<td>F⁺ asnA32 asnB32 thi-1 relA1</td>
<td>E. coli Genetic Stock Center, no. 4813</td>
</tr>
<tr>
<td>C1704*</td>
<td>F⁺ hcr thyA repC val†</td>
<td>R. Calendar</td>
</tr>
<tr>
<td>NK5304</td>
<td>Hfr srl1300::Tn10 ilv318 thr300 spec300 relI recA56</td>
<td>N. Kleckner</td>
</tr>
<tr>
<td>LS108</td>
<td>F⁺ asnA31 asnB32 thi-1 relA1 rep₃ srl300::Tn10 recA56</td>
<td>Two-step P1 transduction: (i) val† rep₃ were cotransduced from C1704 into ER; (ii) srl1300::Tn10 recA56 were then cotransduced from NK5304</td>
</tr>
<tr>
<td>LS130</td>
<td>F⁺ asnA31 asnB32 thi-1 relA1 val† srl300::Tn10 recA56</td>
<td>Two-step P1 transduction: (i) val† was derived from C1704; (ii) srl1300::Tn10 recA56 were then co-transduced from NK5304</td>
</tr>
<tr>
<td>LA-6</td>
<td>F⁺ pro thi lacY gal araΔ766 strR sup⁻</td>
<td>G. Wilcox</td>
</tr>
<tr>
<td>K37</td>
<td>Hfr supD</td>
<td>D. Pratt</td>
</tr>
</tbody>
</table>

* This strain was found to carry a mutation conferring valine resistance which maps in or near the ilv operon.

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Fig. 1. Restriction analysis of M13oriC26 and M13oriC81, showing that the oriC asna plasmid pJS5 is cloned into M13Gori1 in two possible orientations. Lanes: 1, Hae III-digested M13Gori1 RFI; 2, Hae III-digested pJS5 DNA; 3, Hae III-digested M13oriC26 RFI; 4, Hae III-digested M13oriC81 RFI; 5, Hpa II-digested M13Gori1 RFI; 6, Hpa II-digested pJS5 DNA; 7, Hpa II-digested M13oriC26 RFI; 8, Hpa II-digested M13oriC81 RFI. Fragment sizes in lane 1 are determined from DNA sequences of the M13 and G4 genomes (26, 27) and are shown in bp. Doublet bands are indicated by brackets.
Efficient complementation of the asn<sup>−</sup> mutation in both LS130 (rep<sup>+</sup>) and in LS108 (rep<sup>−</sup>) occurred after infection with either M13oriC26 or M13oriC81 phage (Table 2). Infection of LS130 or LS108 with either phage resulted in the efficient production of colonies no longer requiring asparagine. In fact, the ratio of asn<sup>+</sup> colonies to total colony forming units (CFU) upon M13oriC26 or M23oriC81 infection of the rep<sup>−</sup> host was approximately equal to the value for infection of the rep<sup>+</sup> host. Because the host strains carry the recA56 mutation, homologous recombination between the asn gene carried by the phages and the defective gene in the chromosome is not expected. Thus, the data indicate that replication of the chimeric DNAs does occur in the rep<sup>−</sup> host to allow production of a functional asparaginase synthetase.

If a single round of replication of M13oriC26 or M13oriC81 were to occur per host cell generation in the rep<sup>−</sup> host as a result of replication from the M13 origin, the chimeric DNAs could be maintained in the cell, allowing production of colonies no longer requiring asparagine. To demonstrate that the replication that occurs in the rep<sup>−</sup> host is not due to replication from the M13 origin, M13::Tn3-15, an M13 phage carrying the transposable element Tn3 which confers ampicillin resistance, was used to infect LS108 and LS130. The infected cells were plated for ampicillin-resistant colony formation. Ampicillin-resistant CFU were efficiently produced upon M13::Tn3-15 infection of LS130 (rep<sup>+</sup>), whereas no detectable ampicillin-resistant CFU were produced upon infection of LS108 (rep<sup>−</sup>) (Table 2). Because a single copy of the β-lactamase gene per cell is sufficient to confer resistance to ampicillin at 20 μg/ml (28), the data show that replication from the M13 origin does not occur at the level of even one round per host cell generation in LS108. Because duplex replication of M13oriC26 and M13oriC81 from the M13 origin is prevented in the rep<sup>−</sup> host, a functional oriC is apparently responsible for the maintenance of the chimeric DNAs in LS108 (rep<sup>−</sup>). Thus, the data suggest that the cloned oriC allows replication of the chimeric DNAs beyond the rep block.

**Table 2. Genetic assay of oriC function**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>Relevant genotype</th>
<th>Selection</th>
<th>Plating efficiency&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>M13oriC26</td>
<td>LS130</td>
<td>asnA31 recA56</td>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M13oriC81</td>
<td>LS130</td>
<td>asnA31 recA56</td>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.5 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M13oriC26</td>
<td>LS108</td>
<td>asnA31 rep3 recA56</td>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.6 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M13oriC81</td>
<td>LS108</td>
<td>asnA31 rep3 recA56</td>
<td>Asn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.6 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>M13::Tn3-15</td>
<td>LS130</td>
<td>asnA31 recA56</td>
<td>Amp&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1.7 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M13::Tn3-15</td>
<td>LS108</td>
<td>asnA31 rep3 recA56</td>
<td>Amp&lt;sup&gt;−&lt;/sup&gt;</td>
<td>&lt;3.8 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

LS130 and LS108 were grown in LB broth at 37°C with aeration to approximate 10<sup>8</sup> cells per ml. Approximately 6 x 10<sup>6</sup> cells were infected at a multiplicity of infection of 100 with M13oriC26, M13oriC81, or M13::Tn3-15. Cells with or without phage were incubated for 15 min at 37°C and dilutions were plated on minimal glucose plates with or without L-asparagine (100 μg/ml) or on TYE plates with or without ampicillin (20 μg/ml).

<sup>+</sup> Plating efficiency is defined as non-asparagine-requiring colony-forming units (CFU)/ml divided by total CFU/ml or ampicillin-resistant CFU/ml divided by total CFU/ml.
M13oriC26 and M13oriC81 RF in the long-term infected cells would indicate that the DNA is self-replicating in rep+ and rep- hosts. In addition, some indication of the efficiency of replication from oriC in the rep- host is provided by comparison of cleared lysates of LS108 and LS130 infected cells. Lanes 2 and 11 show the above cleared lysate plus 0.1 μg of purified M13oriC81 RFI admixed as a marker; the marker M13oriC81 RFI migrated faster than the host DNA. Lanes 3 and 4 show cleared lysates from two independent asn+ isolates derived from M13oriC26 infected colonies of LS108. Lanes 5 and 6 show cleared lysates of two asn+ isolates of LS108 infected with M13oriC81 phage. In lanes 5–6, a DNA species that comigrated with the marker M13oriC81 RFI in lane 2 was present, indicating that physically detectable levels of cytoplasmic M13oriC26 and M13oriC81 DNA are present in long-term infections of LS108 (rep-). Lanes 7 and 8 show cleared lysates from two independent asn+ isolates of LS130 (rep+) infected with M13oriC26 phage; lanes 9 and 10 show cleared lysates from two independent asn+ isolates of LS130 infected with M13oriC81 phage. Lanes 7–10 show that M13oriC26 RFI and M13oriC81 RFI are present in long-term infections of LS130 (rep+) as indicated by the presence of a DNA species comigrating with the M13oriC81 RFI marker in uninfected LS130 (lane 11).

The minilysate analysis demonstrates that no illegitimate recombination had occurred to allow complementation of the asn- mutation but, instead, that the oriC asnA-containing hybrid DNA molecules were maintained as autonomously replicating species. The experiment provides further support for the conclusion that the cloned oriC functions to allow the chimeric molecules to replicate in the absence of the rep protein. The analysis also provides an indication of the efficiency of replication from oriC in the rep- host. The levels of M13oriC26 and M13oriC81 RFI in the cleared lysates of the infected rep- and rep+ hosts can be compared because each lane represents the extrachromosomal DNA derived from approximately 2 × 106 cells. A single round of replication from oriC per generation (that is, one M13oriC RFI per each of the 2 × 106 cells) would provide approximately 3 ng of M13oriC26 or M13oriC81 DNA per lane in the above experiment. This would not be physically detectable. On the other hand, the amount of M13oriC26 or M13oriC81 DNA present upon analysis of equal amounts of the infected rep- and rep+ cleared lysates is comparable. Because the number of RF molecules per cell upon normal M13 infection is approximately 100 (as would be expected in the infected rep+ host), it is evident that M13oriC26 and M23oriC81 DNAs are present at a substantial copy number in the rep- host.

**DISCUSSION**

Recent studies on ΦX174 RF replication indicate that rep protein of *E. coli* functions in phage replication to unwind duplex DNA in order to allow the single-stranded DNA to serve as template (1, 2). It has been suggested that rep protein also serves in this role during *E. coli* replication. We have tested this possibility by inserting an *E. coli* "mini-chromosome" containing the chromosomal origin and the adjacent asparagine synthetase gene into an M13 phage vector containing an appropriate cloning site.

The experiments presented here indicate that such chimeric molecules containing both the M13 origin and the *E. coli* chromosomal origin can replicate as a phage in a rep+ host, undergoing single-strand DNA synthesis and viral morphogenesis, or as a plasmid in a rep- host. The rep+ mutation prevents replication from occurring from the M13 origin (7). In this case, replication presumably initiates from the *E. coli* chromosomal origin and yields only double-stranded forms of the chimeric DNA. Because these molecules also carry a wild-type asn gene, their presence is confirmed by complementation of the chromosomal asn mutation as well as by direct analysis on agarose gels.

It is of interest to note that the cloned asn gene is expressed in either orientation. The M13 genome is expressed in only a single direction (29) and by a mechanism in which transcripts normally pass through the intergenic space (30–32) where the foreign DNA has been cloned. Insertion of a gene transcribed in the opposite direction could give rise to an inhibition of expression of both the foreign gene and of M13 genes. Such inhibition was not observed. Thus, the utility of M13 as a vector for cloning foreign genes and having them expressed in *E. coli* is not apparently limited by this possibility.

The ability of M13oriC phages to undergo extensive duplex DNA replication in a rep+ host indicates a clear difference in the requirements for rep protein in replication forks initiated at the *E. coli* origin and those initiated at the normal M13 origin. Several possible explanations for this difference may be proposed. (i) Some protein other than the rep protein may serve to unwind the complementary DNA strands ahead of the replication fork when initiation occurs from the *E. coli* origin. In this regard, it should be noted that other ATP-dependent en-
zymes catalyzing DNA strand separation have been discovered in *E. coli* (33–35). (ii) Another unwinding enzyme may substitute for the rep protein in *E. coli* replication forks but not in M13 replication forks. (iii) The rep3 mutation may alter the ability of rep protein to function in asymmetrically replicating molecules in which one side of the replication fork remains single stranded throughout the replication cycle while not affecting its ability to function in replication forks in which both sides of the fork are rapidly converted to a duplex form.

By whatever mechanisms the M13oriC phages escape the inhibition of the *rep*3 mutation, the rep-independent replication of these phages indicates that the viral DNA has acquired replication properties characteristic of the *E. coli* chromosome which is some 400-fold larger. The availability of small phages capable of replicating either by the usual M13 mechanism or by the *E. coli* mechanism will facilitate both biochemical and genetic studies of chromosome replication in *E. coli*. Because the cloned bacterial replication origin is not essential for replication and morphogenesis of the chimeric phage genome, the isolation of mutants defective in initiation from the *E. coli* origin should be feasible. In addition, the use of these viral DNAs as templates for in vitro replication by the bacterial replication mechanism may provide a means for isolating the factors responsible for initiation at the *E. coli* replication origin.

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